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Long-Wavelength Fluorescence from 2-Aminopurine-Nucleobase Dimers in DNA**

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Supporting information:
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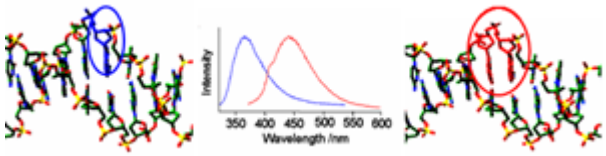
Graphical abstract:



Keywords:
Dimer; DNA; excimer; fluorescence; stacking interaction

Abstract

When 2-aminopurine (2AP) is substituted for adenine in DNA, it is widely accepted that its fluorescence spectrum is essentially unchanged from that of the free fluorophore. We show that 2AP in DNA exhibits long-wavelength emission and excitation bands, in addition to the familiar short-wavelength spectra, as a result of formation of a ground-state heterodimer with an adjacent, π-stacked, natural base. The observation of dual emission from 2AP in a variety of oligodeoxynucleotide duplexes and single strands demonstrates the generality of this phenomenon. The photophysical and conformational properties of the long-wavelength-emitting 2AP-nucleobase dimer are examined. Analogous long-wavelength fluorescence is seen when 2AP π-stacks with aromatic amino acid sidechains in the active sites of methyltransferase enzymes during DNA nucleotide flipping.

Introduction

2-aminopurine (2AP), a close structural analogue of adenine (6-aminopurine), is a highly effective fluorescent probe of DNA structure because its emission properties respond sensitively to local duplex conformation. 2AP can be substituted for adenine without disrupting the structure of the double helix because it forms a Watson-Crick base pair with thymine, and it can be excited selectively, at longer wavelength than the natural bases. 2AP has been widely employed in the study of DNA conformation[1-3], base dynamics[4-8]], interbase electron transfer[9-12] and DNA- and RNA-protein interactions[13-20]. When 2AP is substituted for a natural base in the duplex its fluorescence is strongly quenched. A primary quenching mechanism is charge transfer between excited 2AP and neighboring bases, most favorably electron transfer from guanine to 2AP. Time-resolved fluorescence studies of 2AP-labeled DNA show that the duplex exists in a multiplicity of conformational states, manifested by a complex decay that can be described by the sum of four exponential components[4-6,8,17,21] with typical lifetimes of <100ps, ~0.5ns, ~2ns and ~10ns. It is generally accepted that the very short component (<100ps) corresponds to a highly stacked conformation in which excited 2AP is rapidly quenched by interbase charge transfer[22,23]. The long, ~10ns, lifetime is attributed to an unstacked conformation in which 2AP is extrahelical[4,5,17,21,24]. The intermediate lifetimes correspond to partially stacked structures between these two extremes.

Typically, 2AP-containing duplexes are excited between 300 and 320 nm giving rise to an emission spectrum that closely resembles that of 2AP in solution and is widely accepted as the characteristic fluorescence of 2AP in DNA. However, recently we reported preliminary results for a single duplex showing that 2AP in DNA exhibits a second, red-shifted emission band when excited at 360 nm[25]. 2AP in solution does not show this long- wavelength emission. We now report a comprehensive study of ten 2AP-containing duplexes and four 2AP-containing single-strand oligodeoxynucleotides (see Table 1), in which we demonstrate that dual

fluorescence is a general property of 2AP in DNA and explore the nature of the 2AP-nucleobase interaction that gives rise to long-wavelength emission.

Table 1. Base sequences of the DNA duplexes.

Duplex[a]	Base Sequence[b]
APT(T)	5'-AGACGA**P**TTTCGAA-3' 3'-TCTGCTTAAAGCTT-5'
APC(T)	5'-CACGGGCCTA**P**CGATATCGTGCGTACGAGC-3' 3'-GTGCCCGGATTGCTATAGCACGCATGCTCG-5'
TPT(T)	5'-GATCGTAGAT**P**TCGCATCGA-3' 3'-CTAGCATCTATAGCGTAGCT-5'
GPT(T)	5'-GATCGTAG**P**TATCGCATCGA-3' 3'-CTAGCATCTATAGCGTAGCT-5'
GPA(T)	5'-TGCAG**P**ATTCGAGGTCGACGG-3' 3'-ACGTCTTAAGCTCCAGCTGCC-5'
GPC(T)	5'-TGCAGAATTCGAGGTCGACGG-3' 3'-ACGTCTTAAGCTCC**P**GCTGCC-5'
GPG(T)	5'-GACAGTATCAGGCGCCTCCCCACAA-3' 3'-CTGTCATAGTCCGCGG**P**GGGGTGTT-5'
APA(C)	5'-TGCA**P**AATTCGAGGTCGACGG-3' 3'-ACGTCTTAAGCTCCAGCTGCC-5'
GPT(C)	5'-TGCAGPATTCGAG**P**TCGACGG -3' 3'-ACGTCTTAAGCTCCAGCTGCC-5'
GPG(G)	5'-ACTGGTACAGTATCAGG**P**GCTGACCCACAACATCCG-3' 3'-TGACCATGTCATAGTCCGCGACTGGGTGTTGTAGGC-5'

[a]Duplexes are designated XPY(Z) where P is 2AP, X and Y are the bases stacked with 2AP in the 5'and 3' position, respectively, and Z is paired with 2AP.

[b]2AP is shown as **P** in the base sequence.

Investigation of the emission of 2AP-containing DNA at excitation wavelengths longer than those commonly used was prompted by our observation that crystalline 2AP has excitation and emission spectra (λ_{max} 370 nm and 420 nm, respectively) that are red-shifted relative to those of 2AP in solution (λ_{max} 300 nm and 370 nm, respectively), as a result of π-stacking interaction between 2AP molecules in the crystal lattice[25]. Extended columns of π-stacked molecules are present in the 2AP crystal, resembling the stacked bases in DNA. The separation between adjacent stacked molecules in the crystal is 3.3 Å, compared with the average rise of 3.4 Å between base steps in DNA, as determined by El Hassan and Calladine in a survey of 400 steps from 60 crystal structures[26]. The values of roll, tilt, shift and slide parameters in the 2AP stacks are also very similar to those found in for DNA base steps. However, a significant difference is that in the 2AP crystal there is no twist (rotational displacement about the stack axis) between adjacent molecules in the π-stack, whereas in classical B-form DNA there is a helical twist of 36^{o} between successive base pairs, and the average value

reported by El Hassan and Calladine[26] is 35°. Thus, there is less π-overlap between 2AP and neighbouring bases in the B-form duplex than there is between stacked 2AP molecules in the crystal, so that the predominant emission of 2AP in DNA resembles that of the free fluorophore in solution. As we proposed previously[25] and discuss in more detail below, the observation of long-wavelength emission from 2AP in DNA implies the existence of conformational states in which the helical twist is less than in the B-form structure, such that the π-overlap between 2AP and an adjacent base approaches that found in the highly eclipsed stacks in the crystal lattice.

There is some previous evidence of long wavelength fluorescence from 2AP in DNA. Rist *et al.*[27] reported weak red-shifted excitation and emission bands for a duplex containing four separate 2AP bases. This was attributed to exciplex-type emission as a result of ground-state excitonic interaction of 2AP with other adjacent DNA bases. Prior to this, long wavelength emission had been observed by Guest *et al.* for singly 2AP-labelled 7-mer oligonucleotide duplexes and was attributed to exciplex formation between 2AP and guanine[5]. In this instance 320-nm excitation produced sufficient red-shifted fluorescence to noticeably distort the 2AP emission profile.

The excited-state properties of 2AP stacked with other nucleobases in di- and trinucleotides of B-form structure have been investigated theoretically.[28-31] Jean and Hall, [28,29] using time-dependent density functional theory (TDDFT), predicted that stacking of 2AP with other bases leads to the formation of excited states of charge-transfer (CT) character energetically below a 2AP-like transition. The low oscillator strengths calculated for the CT transitions led to the conclusion that the CT states would be dark states and fluorescence would originate from the higher energy excited state localized on 2AP. Internal conversion from the emitting state to the lower energy CT states was proposed as a mechanism of efficient quenching of 2AP fluorescence in DNA. Recently, Hardman and Thompson[30] have pointed out that the results of Jean and Hall are questionable because many (or perhaps even all) of the low energy CT states predicted by TDDFT are spurious. They report the results of calculations using the configuration interaction singles (CIS) method that predict that, in all cases, the lowest energy transition of 2AP stacked with other bases is of $\pi\pi^*$ character with energy essentially the same as that of free 2AP, but with somewhat reduced oscillator strength, as a result of some delocalization of the participating π orbitals over the other bases.[30,31]

Electronic coupling between the natural bases in DNA, as manifested by excimer formation, has attracted great interest because of its relevance to charge transport[32] and the possible involvement of long-lived excited dimer states in the formation of UV-induced photolesions, including photodimers[33]. Excimer emission from DNA was first discovered in low temperature fluorescence experiments by Eisinger *et al.* in 1966[34]. For dinucleotides, polynucleotides and DNA, they reported fluorescence red-shifted relative to the corresponding absorption spectrum and the emission spectra of mononucleotides. This has been confirmed by various subsequent observations, as summarised in references 35-37. Although the habitual use of the term "excimer" to describe the emitting state implies an excited dimer that is formed dynamically following local excitation of

one of the constituent monomers, there has been little evidence to support this assumption. There is great uncertainty about the nature of both the initially excited Franck-Condon excited state and the long-lived excited state responsible for excimer emission. The extremely low fluorescence quantum yield of the nucleic bases severely inhibits conventional spectroscopic studies and the definitive measurement of the excitation spectrum of the excimer emission remains elusive. The only report of such a measurement in solution at room temperature appears to be that of Konova and Bukina[38] for polyadenylic acid (poly(A)); their excitation spectrum of the red-shifted emission of poly(A) shows a weak shoulder at ~320 nm on the long-wavelength edge of the intense 260-nm monomer absorption band, providing evidence of a bound (electronically coupled) dimer ground state. Recent femtosecond transient absorption[33,35,36] and femtosecond time-resolved fluorescence[35] studies of poly(deoxyadenosine) ($(dA)_n$) oligonucleotides have focused on the mechanism of excitation of long wavelength emission via the intense 260-nm absorption band. These all show that 260-nm excitation populates an adenine-like excited state that relaxes on the sub-picosecond timescale to a relatively long-lived (~150 ps) delocalised excited state that emits red-shifted fluorescence.

To avoid the complexity of interrogating a heterogeneous excited state population, produced by simultaneous excitation of the strongly overlapping absorption bands of the natural bases, studies of excimer formation in DNA have been restricted mainly to single-strand oligonucleotides composed of a single type of base, usually adenine. In contrast, the ability to selectively excite 2AP, together with its relatively high fluorescence quantum yield, have made it straightforward to show unequivocally that this close analogue of adenine forms an electronically coupled ground-state dimer with each of the natural bases in duplex DNA, as will now be described.

Results

Long-wavelength fluorescence from duplexes at room temperature.

The sequences of the 2AP-containing duplexes studied are given in Table 1. On excitation at 360 nm, all of the duplexes showed a long-wavelength emission band, with maximum intensity at ~450 nm. This is illustrated for duplex GPA(T) in Figure 1, where the long-wavelength emission spectrum is shown in comparison with the familiar short-wavelength spectrum, excited at 310 nm. The long-wavelength emission is relatively weak, only about 10% of the intensity of the 370-nm emission band for this duplex.

The relative intensity of the long-wavelength band depends on the sequence context of the 2AP, as shown in Table 2, ranging from only 3% in duplexes APA(C) and GPT(C) to 32% in duplex TPT(T). The sequence-dependence of the intensity of each emission band is shown in Table 3. It can be seen that the intensity of the short-wavelength emission is affected much more by sequence context than is that of the long-wavelength band. It is apparent that the higher relative intensity of long-wavelength emission in duplexes GPC(T),

GPG(T), GPG(G), GPT(T) and TPT(T) is mainly the consequence of efficient quenching of the 370-nm fluorescence, rather than a greater absolute intensity of the 450-nm band. In addition to the data shown, which includes duplexes in which 2AP is stacked between pairs of A, G and T, we have qualitatively observed 450-nm emission from a duplex with sequence context CPC(T). Thus, long-wavelength emission is observed when 2AP stacks with any of the natural bases.

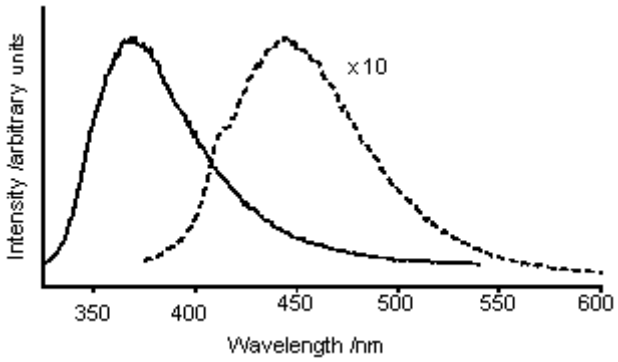


Figure 1. Emission spectra of the GPA(T) duplex, excited at 310 nm (solid line) and 360 nm (broken line, intensity multiplied by a factor of 10; the shoulder at 410 nm is a Raman band of water).

Table 2. The relative intensity of the long-wavelength (450-nm) emission for duplexes in solution at room temperature.

Duplex	Relative intensity [a]
APA(C)	0.03
GPT(C)	0.03
APT(T)	0.04
APC(T)	0.05
GPA(T)	0.11
GPC(T)	0.22
GPG(T)	0.24
GPG(G)	0.27
GPT(T)	0.28
TPT(T)	0.32

[a] Intensity of the 450-nm emission, excited at 360 nm, relative to that of the short-wavelength (370-nm) emission, excited 310 nm.

Table 3. The intensity of each emission band relative to that of GPT(C) for duplexes in solution at room temperature.

Duplex	370-nm band[a]	450-nm band[b]
APA(C)	0.9	1.0
GPT(C)	1.0	1.0
APT(T)	0.6	0.7
APC(T)	0.2	0.3
GPA(T)	0.2	0.6
GPC(T)	0.08	0.6
GPG(T)	0.07	0.6
GPG(G)	0.08	0.8
GPT(T)	0.03	0.3
TPT(T)	0.02	0.2

[a]The intensity of the 370-nm emission, excited at 310 nm, relative to that of GPT(C).

[b] The intensity of the 450-nm emission, excited at 360 nm, relative to that of GPT(C).

The duplexes are listed in order of increasing relative intensity of long-wavelength emission, as in Table 2.

The excitation spectra of the short- and long-wavelength fluorescence are shown in Figure 2. The excitation maximum of the long-wavelength emission is at ~360 nm. The excitation spectrum recorded at an emission wavelength of 450 nm shows a peak at ~310 nm, coinciding with the excitation peak of the short-wavelength emission, in addition to the 360-nm peak. Only a fraction of the intensity of the 310-nm excitation band is due to the long-wavelength emission. It contains a large contribution from the tail of the (relatively intense) 370-nm emission band that extends into the long-wavelength region, as demonstrated in Figure 3, where excitation spectra recorded at progressively longer emission wavelengths show decreasing relative intensity of the short-wavelength excitation band. Nevertheless, in some cases, the 310-nm excitation of long-wavelength emission can significantly distort the "normal" 2AP emission profile, as shown in Figure 4, where the emission spectra of duplexes GPT(C) and GPG(G) are compared. As indicated in Table 2, the relative intensity of 450-nm emission from duplex GPG(G) is high, whereas that for GPT(C) is low. Excitation of GPG(G) at 310 nm gives an emission spectrum which is substantially broadened to the red, compared with that of GPT(C). Using the GPT(C) spectrum as an indication of the upper limit of the contribution of the short-wavelength tail to the emission intensity at 450 nm, we estimate that about 40% of the intensity of the 310-nm excitation band (450-nm emission) of GPA(T) in Figure 2 is due to long-wavelength emission.

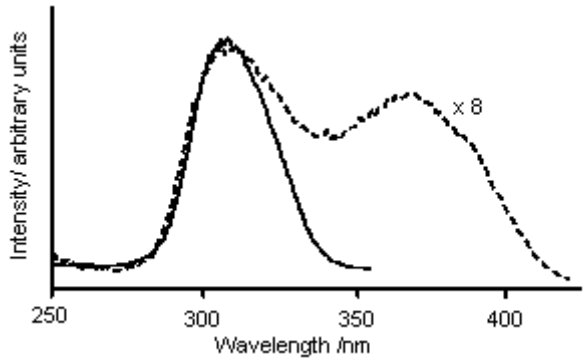


Figure 2. Excitation spectra of the GPA(T) duplex, detected at 370 nm (solid line) and 450 nm (broken line, intensity multiplied by a factor of 8; the shoulder at 390 nm is a Raman band of water).

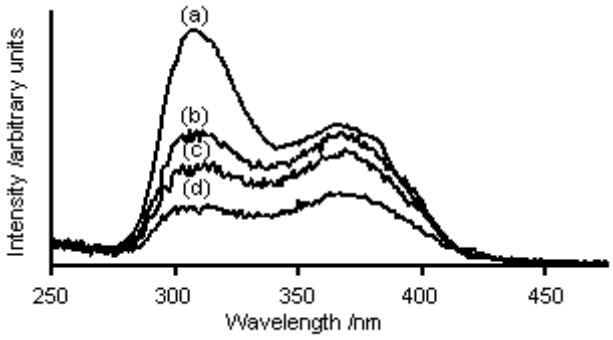


Figure 3. Excitation spectra of the GPA(T) duplex, detected at (a) 440 nm, (b) 460 nm, (c) 470 nm and (d) 490 nm.

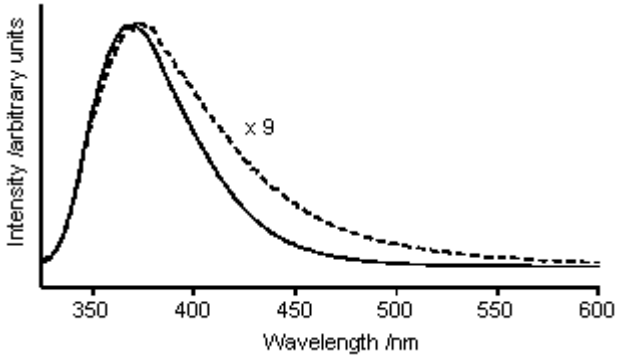


Figure 4. Comparison of the emission spectra of GPT(C) (solid line) and GPG(G) (broken line, intensity multiplied by a factor of 9), excited at 310 nm.

In the UV absorption spectrum of 2AP-labelled duplexes (see, for example, Figure S1 of the Supporting Information), the short-wavelength (310-nm) absorption band appears as a shoulder on the long-wavelength edge of the much more intense absorption band of the natural bases. There is no distinct absorption band visible at 360 nm, but a very weak long wavelength tail of the 310-nm band extends into this region. This places an upper limit on the absorbance of the long-wavelength emitting species at 360 nm as being <10% of the absorbance of the short-wavelength emitting species at 310 nm.

Decay functions of the long-wavelength fluorescence were recorded for three of the duplexes. The decays are multiexponential, requiring 4 lifetimes to give a satisfactory fit. As discussed above, such heterogeneous decays are well established for the short-wavelength emission of 2AP-containing duplexes. The lifetimes and A factors are given in Table 4, together with those for the short-wavelength decays. The decay times at 450 nm are generally significantly longer than those at 370 nm. The 450-nm decay parameters (both lifetimes and A factors) show little variation with sequence context, in contrast to the 370-nm parameters; this is particularly notable for the shortest component, whose lifetime (τ_1) and amplitude (A_1) are strongly dependent on sequence for the 370-nm emission.

Table 4. Fluorescence decay parameters for duplexes at emission wavelengths of 450 nm and 370 nm, in solution at room temperature.

Duplex		τ_1 / ns	τ_2 / ns	τ_3 / ns	τ_4 / ns	A_1	A_2	A_3	A_4
APT(T)	450 nm	0.15	1.0	3.5	11.5	0.49	0.29	0.17	0.05
	370 nm	0.15	0.46	2.5	9.9	0.51	0.36	0.04	0.08
APC(T)	450 nm	0.17	0.74	3.6	12.6	0.44	0.38	0.13	0.05
	370 nm	0.12	0.37	2.7	10.8	0.88	0.08	0.02	0.02
GPG(T)	450 nm	0.19	0.97	3.6	9.2	0.53	0.26	0.16	0.05
	370 nm	0.04	0.44	2.7	10.7	0.69	0.11	0.09	0.11

[a] 450-nm fluorescence decays were excited at 360 nm.

[b] 370-nm fluorescence decays were excited at 320 nm.

Long-wavelength fluorescence from duplexes at 77K

To explore the influence of base dynamics on the long-wavelength emission, a selection of the duplexes were frozen in 10M LiCl solution at 77K. Previously, this approach has demonstrated that elimination of base motion leads to a marked increase in the intensity of the short-wavelength emission[8,9]. As shown in Table 5, freezing the duplex has a similar effect on the intensity of the 450-nm fluorescence.

The emission spectra of the APT(T) duplex at 77K are shown in Figure 5, in comparison with the corresponding spectra at 298 K. Both short and long wavelength bands are shifted to the blue at 77K, a

commonly observed effect of arresting solvent relaxation. In the low-temperature emission spectrum excited at 310 nm, the 450-nm band is evident as a distinct shoulder above 430 nm.

Table 5. The intensities of the long- and short-wavelength emission bands for duplexes at 77 K, relative to their respective intensities at 298 K.

Duplex	370-nm band[a]	450-nm band[a]
APT(T)	6	7
GPC(T)	4	4
GPG(G)	6	7

[a] Intensity for the duplex frozen in optically transparent LiCl glass at 77 K, relative to that in the same LiCl solution at 298 K.

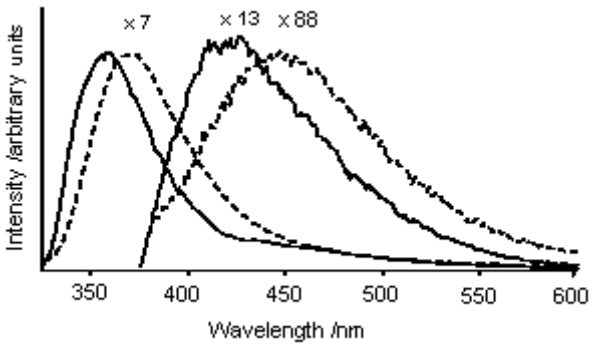


Figure 5. Emission spectra (solid lines) of the APT(T) duplex frozen at 77K, excited at 310 and 360 nm. The corresponding spectra at 298 K are also shown (broken lines). Intensities have been multiplied by the factors shown.

Long wavelength fluorescence from single-strand oligodeoxynucleotides at room temperature

Four of the 2AP-containing oligodeoxynucleotides, APA, GPA, GPC and TPT (sequences as shown in Table 1), were investigated as single strands. As shown in Table 6, they all show long-wavelength emission with intensity that is a few percent of the 370-nm band. The high relative intensity of 450-nm fluorescence seen for duplexes GPC(T) and TPT(T) (Table 2) is not reproduced in the single strands. This is because the extreme quenching of 370-nm fluorescence found in these duplexes (Table 3) does not occur in the single stands, as shown in Table 7. The inter-oligonucleotide variation in the intensity of the 370-nm emission is comparable to that of the 450-nm emission.

Table 6. The relative intensity of the long-wavelength (450-nm) emission for single-strand oligodeoxynucleotides in solution at room temperature.

Oligo	Relative intensity [a]
APA	0.01
GPA	0.05
GPC	0.05
TPT	0.01

[a] Intensity of the 450-nm emission, excited at 360 nm, relative to that of the short wavelength (370-nm) emission, excited 310 nm.

Table 7. The intensity of each emission band relative to that of APA for single-strand oligodeoxynucleotides in solution at room temperature.

Oligo	370-nm band[a]	450-nm band[b]
APA	1.0	1.0
GPA	0.4	1.5
GPC	0.2	0.8
TPT	0.8	0.5

[a]The intensity of the 370-nm emission, excited at 310 nm, relative to that of APA

[b]The intensity of the 450-nm emission, excited at 360 nm, relative to that of APA.

Discussion

We attribute the long-wavelength emission from 2AP in DNA to conformational states of the duplex in which 2AP forms a highly overlapped, π-stacked heterodimer with one of its stacking partners. The electronic interaction between 2AP and its partner base in this nucleodimer gives rise to an excited state of substantially lower energy than the lowest locally excited state of 2AP. The observation of a corresponding long-wavelength excitation spectrum indicates that the nucleodimer is bound (electronically coupled) in the ground state. This concurs with the previous findings of Rist *et al.*,[27] who also inferred the existence of ground-state interaction between 2AP and neighbouring bases in duplex DNA. The observation of long-wavelength emission in single-strand oligodeoxynucleotides confirms that it arises entirely from base stacking interaction.

The familiar short-wavelength emission of 2AP-containing DNA originates from conformations in which the electronic structure of 2AP is essentially unperturbed by base-stacking interactions (although the rate of non-radiative decay of the excited state can be greatly increased by such interactions). We suggest that the essential difference between the short-wavelength and long-wavelength emitting conformations is the size of the helical twist (rotational angle about the long axis of the helix) between 2AP and its flanking bases, since this is the step parameter that limits the π-overlap between successive bases in B-form DNA. In a fraction of

the conformational population (perhaps only a few percent, as discussed below) the twist between 2AP and one of its stacking partners is less than some critical value, such that there is sufficient π-overlap for 2AP nucleodimer formation and consequent long-wavelength emission. For conformations with twist angles greater than this critical value, we observe short-wavelength emission. Hereafter the short-wavelength emitting species will be called 2AP monomer.

Quantum chemical calculations[29-31] predict that 2AP stacked with two flanking nucleobases in the B-form structure will fluoresce at a similar wavelength to the free molecule and this is consistent with the experimentally observed predominance of short-wavelength emission from 2AP in DNA. Thus, nucleodimer formation can be assumed to require greater overlap than is found in the equilibrium geometry of the B-form duplex. Figures 6 (a) and (b) show details from the crystal structure of a duplex in which 2AP is stacked between two Gs and paired with T, to illustrate the overlap between 2AP with its 5' and 3'neighbours.[39] Figure 6(c) illustrates a putative, highly eclipsed, nucleodimer structure that can be formed by reducing the twist between 3'G and 2AP in this duplex.

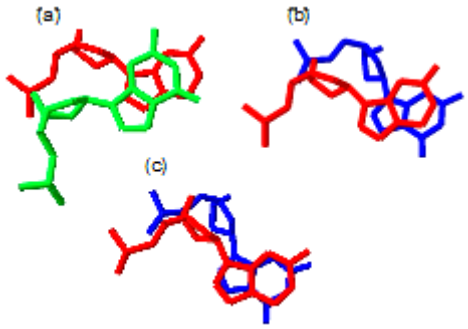


Figure 6. Detailed views from the crystal structure of a duplex with 2AP in the context GPG(T), looking down the long axis of the helix, to illustrate the overlap of (a) 2AP (red) with 5'G (green) and (b) 2AP (red) with 3'G (blue). A hypothetical structure of a 2AP-G nucleodimer, obtained by decreasing the twist between 2AP and 3'G, is shown in (c).

The most highly populated short-wavelength emitting conformation, which is characterized by a very short lifetime τ_1), is generally described as a "highly stacked" state, susceptible to rapid charge transfer quenching. In this conformation the overlap between 2AP and its flanking bases is evidently sufficient for efficient charge transfer, but insufficient for nucleodimer formation, since the emission wavelength is characteristic of 2AP monomer. As shown previously,[8] this highly quenched 2AP monomer conformation is accessed by thermal motion of the bases and is not populated in the frozen duplex at 77K. In contrast, the persistence of long-wavelength emission at 77K demonstrates that formation of the nucleodimer does not require thermal motion

of the bases and this stacking arrangement constitutes one of the minimum energy structures of the duplex. The highly stacked short-wavelength emitting conformation can be envisaged as lying intermediate between the B-form geometry and the nucleodimer geometry along the twist coordinate, as illustrated in Figure 7, and can be populated by thermal excursions from either of these equilibrium geometries.

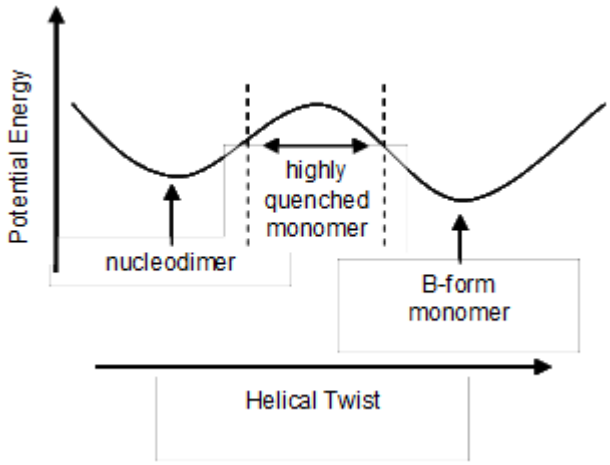


Figure 7. Schematic illustration of the conformational relationship between the B-form 2AP monomer, the 2AP nucleodimer and the highly quenched 2AP monomer, in terms of the helical twist coordinate.

For duplexes APT(T), APC(T) and GPG(T) (Table 4), the mean lifetimes ($\Sigma A_i\tau_i/\Sigma A_i$) of the 450-nm emission are very similar 1.44, 1.45 and 1.39, respectively, showing that the quantum yield is essentially independent of sequence for these duplexes, if we assume that the radiative lifetime (oscillator strength) of the nucleodimer transition does not depend significantly on 2AP's stacking partner. However, the intensity of 450-nm emission from APT(T) is more than twice that of APC(T), indicating that the population of nucleodimers in APT(T) is more than twice that in APC(T). If we assume that the invariance in quantum yield can be extrapolated to all of the duplexes, then the inter-duplex 450-nm relative intensities in Table 3 reflect the relative populations of the nucleodimer-forming conformations. The highest population of nucleodimers is found in duplexes in which 2AP is base-paired with C (wobble base pair, see Figure S2 of the Supporting Information) or G (Hoogsteen-type base pair, see Figure S3 of the Supporting Information) rather than with T (Watson-Crick base pair, see Figure S3 of the Supporting Information). This is consistent with the notion that access to nucleodimer conformations will be facilitated by increased freedom of the 2AP base to twist about the helix axis. The duplex with the lowest nucleodimer population is TPT(T), in which 2AP is substituted for A in the internal position of the ATAT sequence. Previously, time-resolved fluorescence anisotropy measurements have shown that 2AP in this context is highly rotationally constrained and immobile within the base stack.[2] 2AP in ATAT appears to be extremely well stacked; it is resistant to acrylamide quenching, subject to

efficient excitation energy transfer from nearby bases[2] and this is the only duplex we have studied for which the short-wavelength fluorescence decay shows no component (τ_4) due to extrahelical 2AP (unpublished data). However, the nature of the stacking geometry does not favour 2AP nucleodimer formation.

In contrast to the relatively constant 450-nm intensity, the considerable inter-duplex variation in the 370-nm intensity reflects the sequence-dependence of the quantum yield of this emission. In duplexes where 2AP interacts closely with G there is efficient charge-transfer quenching of the short wavelength emission, manifested by low intensity and the presence of a very short lifetime (<100 ps) with large amplitude in the fluorescence decay, as exemplified by the 370-nm decay parameters of GPG(T) in Table 4 This, in turn, is reflected in the sizeable inter-duplex variation in the ratio of the intensities of long and short wavelength emission (Table 3). Thus, duplexes APT(T) and GPG(T), for example, show a factor of 6 difference in their 450nm:370 nm fluorescence intensity ratio, although they have similar populations of 2AP nucleodimers, because of the large difference in their 370-nm quantum yields.

For duplex APT(T), the mean lifetimes of the short- and long-wavelength fluorescence have similar values, 1.18 ns and 1.55 ns. If the radiative lifetimes (oscillator strengths) of the two emissions were of similar magnitude, this would imply that they have similar quantum yields and the relative intensity of long- and short-wavelength fluorescence would indicate a population of the dimer species of about 3% in this duplex. The inter-duplex 450-nm relative intensity values would then suggest that the fractional population of the nucleodimer lies between about 1% and 4% across all the duplexes. A fractional population of a few percent for the nucleodimer, in combination with a comparable oscillator strength to the monomer, would also be consistent with the value of <10% for the absorbance of the dimer relative to the monomer. In this picture, the 2AP monomer is by far the predominant species with a near-constant population of >95% across all the duplexes and the inter-duplex variation in 370-nm intensity is due entirely to the dependence of the quantum yield on 2AP sequence context. If, however, the oscillator strength of the dimer were significantly less than that of the monomer, the population of the dimer would have to be substantially greater to account for the relative intensity of the long wavelength emission and could constitute a substantial fraction of the total 2AP population.

The multiexponential decay of the long-wavelength emission indicates that the nucleodimer exists in a multiplicity of conformational states that are distinguished by different non-radiative decay rates. Conformational heterogeneity of the dimer has a number of possible origins: (i) in each duplex, 2AP may form a nucleodimer with either its 5' or its 3' neighbour; (ii) there will be a range of twist angles (relative orientations of 2AP and its partner base) over which the nucleodimer remains bound; (iii) the nucleodimer as a whole may adopt a range of conformations relative to its neighbouring bases in the duplex. We assume that quenching of the excited dimer occurs through interaction with neighboring bases and hence variation in the non-radiative decay rate arises primarily from (iii). Thus the heterogeneous decay times of the dimer, like those of the monomer, can be interpreted in terms of the geometric relationship between the dimer as a whole

and the local duplex structure (while recognizing that each decay time must represent an ensemble of conformational states with similar quenching rates). The shortest long-wavelength decay time (~ 150 ps) can be attributed to conformations in which the nucleodimer is maximally stacked with its neighbouring bases. The longest decay time is due to conformations in which 2AP remains closely stacked with one of its flanking bases, maintaining the integrity of the nucleodimer, but there is minimal interaction between the dimer and other bases.

The general similarity in magnitudes of the fluorescence lifetimes, τ_1 to τ_3, of monomer and nucleodimer indicate similar non-radiative decay rates and would seem to suggest that both species are subject to similar quenching mechanisms. However, a notable difference is the lack of extremely rapid quenching of the dimer when in close proximity to G. We have shown previously[8] that the conformations in which 2AP monomer is rapidly quenched can be attained only through thermal motion of the bases, even when 2AP is stacked with G. Therefore, the absence of rapid quenching of the dimer by G may be because of the inability to achieve the necessary geometrical relationship, rather than an intrinsic difference in quenching mechanism. On freezing the duplexes at 77K, the dimer shows a comparable increase in emission intensity to the monomer, indicating a similar reduction in quenching rate when conformational fluctuations of the duplex are suppressed.

Comparison of the amplitudes of the decay components (Table 4) indicates that, in general, the population of highly quenched conformations (A_1) is smaller for the dimer than the monomer and the population of moderately quenched conformations (A_3) is much higher. For the dimer, as for the monomer, the population of least quenched conformations (A_4) is very small. In the latter conformations, 2AP monomer is essentially free from interbase quenching, exhibiting a lifetime very similar to that of the free riboside in solution, and is thus envisaged to be extrahelical. We cannot say whether the nucleodimer is able to attain an equally unquenched arrangement, but its τ_4 value gives a lower limit for the intrinsic, unquenched decay time.

In addition to the resonant excitation band at 360 nm, the nucleodimer has a higher energy excitation band at 310 nm, coincident with the S_0 to S_1 transition of the 2AP monomer. This could be due to a higher lying, 2AP-like, excited state of the nucleodimer or could indicate dynamic exciplex formation, through nuclear rearrangement following excitation of the monomer. When conformational relaxation is arrested, in the frozen duplex at 77K, the 310-nm excitation band of the nucleodimer emission persists. This argues against dynamic exciplex formation and supports the direct excitation of a higher energy (static) dimer state. This does not rule out dynamic exciplex formation at room temperature, which would most probably occur from the highly stacked monomer conformation. However, since the latter conformation constitutes 60-90% of the excited monomer population, we would expect the 310-nm nucleodimer excitation band to be much more intense if exciplex formation were a significant decay channel of the monomer. Therefore, the 310-nm excitation band appears to be due primarily to a transition from the dimer ground state. The similarity in intensity of the 360-nm and 310-nm excitation bands indicates that these transitions have comparable oscillator strengths.

On a slightly different note, we draw attention to evidence of long-wavelength emission from extrahelical 2AP in studies of DNA nucleotide flipping by methyltransferase enzymes. Su *et al*[40] reported mysterious red-shifted emission and excitation spectra for 2AP-labelled DNA in complex with the methyltransferase enzyme, M.EcoKI. The observation of long-wavelength fluorescence coincided with nucleotide-flipping of 2AP into the enzyme active site and examination of mutant enzymes showed that it depended on the location of an aromatic amino acid residue (phenylalanine 269) in the active site. Subsequently, we have observed red-shifted fluorescence from a crystalline DNA-enzyme complex in which 2AP is flipped into the active site of the methyltransferase, M.TaqI. The crystal structure of this ternary complex, in which a M.TaqI molecule is bound to a cofactor analogue and a 2AP-labelled 10-mer duplex (with 2AP positioned at the target site of the enzyme), has been reported previously [18]. As shown in Figure 8, the intensity of the 450-nm emission is relatively high, about one third that of the 370-nm emission. The crystal structure[18] shows that the flipped 2AP is π-stacked with the aromatic (phenol) sidechain of tyrosine 108, with which it can apparently form a heterodimer. The observations of Su *et al.* can be explained by analogous heterodimer formation between 2AP and phenylalanine. Thus, long-wavelength emission may prove a useful indicator of the location of the flipped nucleotide in the enzyme active site.

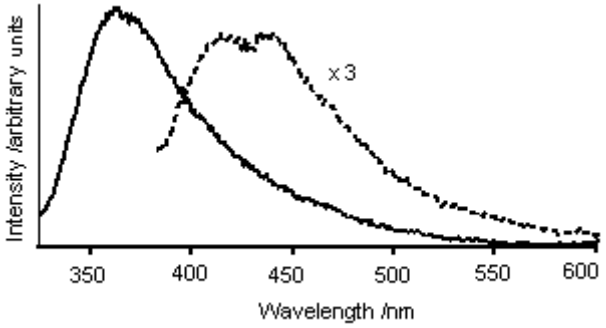


Figure 8. Emission spectra, excited at 310 nm (solid line) and 360 nm (broken line, intensity multiplied by a factor of 3), of a crystalline complex of a 2AP-labeled duplex with the M.TaqI methyltransferase enzyme, in which 2AP is flipped into the enzyme active site.

Conclusion

In the DNA duplex, 2AP exists either as a monomer or a nucleodimer, depending on the extent of π-overlap with an adjacent base in the same strand, which is governed primarily by the helical twist coordinate. Nucleodimer formation, manifested by long-wavelength emission, occurs in duplex conformations where the twist angle between 2AP and one of its stacking partners lies below some critical value. Conformations with

greater twist angles, which include the B-form equilibrium geometry (twist angle 35°), show short-wavelength monomer emission. The nucleodimer is bound in the ground state and can be formed between 2AP and any of the natural bases. Nucleodimer emission is weak compared with monomer emission and in many cases is apparent only if excited selectively via its resonant transition at ~360 nm. The higher energy excitation band of the dimer, at ~310 nm, is due primarily to a transition from the dimer ground state rather than dynamic exciplex formation.

Finally, we consider how close an analogy can be drawn between the long-wavelength emission of the 2AP nucleodimer and the excimer emission of DNA. We argue that the interbase, dimer-forming interaction of 2AP in DNA mimics that of the natural bases and the only significant difference in properties lies in the oscillator strength of the transition connecting the dimer ground state with the long-wavelength-emitting excited state. Ultrafast studies of poly(deoxyadenosine) ($(dA)_n$) oligonucleotides[33,35,36] show that population of the relatively long-lived excimer-like state occurs within less than 1 ps following excitation of the Franck-Condon state at 260 nm, allowing little scope for significant conformational rearrangement. Thus, the stacked dimeric structure must be pre-formed in the ground state, as it is in the 2AP nucleodimer. Transient absorption measurements[33] indicate that a substantial fraction of the stacked conformational population of $(dA)_n$ has a propensity for excimer formation; however, the long-wavelength emission is not much more intense than the monomer emission, in spite of the much (~100x) longer lifetime of the emitting state. This leads to the conclusion that the oscillator strength of the long-wavelength transition is very low.[33,35] A recent theoretical study of the excited states of the stacked dimer of 9-methyladenine[42] predicts that in the Franck-Condon region the S_1 and S_2 states have vanishingly small oscillator strengths and the optical absorption transition at ~260 nm is to the S_3 state, the dimer counterpart of the π^*L_a bright excited state of the monomer. Fast transfer is then predicted to occur from the bright state to an underlying excited dimer state (S_1) with charge transfer character and very low oscillator strength, without any substantial conformational rearrangement. In contrast, in the 2AP nucleodimer the transitions from the ground state to the long-wavelength-emitting state and the higher energy, monomer-like, excited state have comparable oscillator strengths and both are observed. Therefore, the excitation spectrum discloses the ground-state electronic interaction between 2AP and its dimer partner, whereas the analogous interaction between stacked pairs of natural bases evades spectroscopic observation.

Experimental Section

Materials

Oligonucleotides were obtained from commercial sources (Fermentas (Lithuania), MWG Biotech (Germany), Midlands DNA (USA)) or synthesized as described previously.[18] To prepare the duplexes, the 2AP-containing strand at a concentration of 20 μM was combined with a 50% excess of the unlabelled strand to

avoid the presence of unpaired 2AP. The DNA was buffered (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and annealed by heating to 95 ^{o}C and then left to cool slowly to room temperature. Single strands were prepared at a concentration of 20 μM in the same buffer.

Steady-state fluorescence

Steady-state fluorescence spectra were measured using a Fluoromax (Horiba Jobin Yvon) photon-counting spectrofluorometer. Spectra were recorded with a bandpass of 5 nm for both excitation and emission monochromators and were corrected for variation in the excitation lamp intensity. The detector response was constant over the emission wavelength range used (320 to 600 nm). Spectral intensities were determined by integration over a 20-nm bandwidth encompassing the emission maximum. For measurements at room temperature, the sample solution (20 μM duplex or single strand in buffer) was contained in a fused silica cuvette (Starna Ltd.) with capacity 100 μl and path length 1 cm. The fluorescence intensities of the different duplexes were measured under conditions of equivalent absorbance at 310 nm. There was no detectable absorption band at 360 nm and the absorbance at this wavelength was too low to be quantified reliably, at the duplex concentrations available. The absorbance at 360 nm was <10% of that at 310 nm. For measurements at 77 K, duplexes were dissolved in 10 M LiCl (concentration ~ 3.3 μM). The LiCl solution was contained in a cylindrical fused silica tube which was immersed in liquid nitrogen in a fused silica dewar, to give a frozen, optically transparent glass. Measurements on DNA-enzyme crystals were performed by suspending the crystals above buffer solution in a fused silica capillary. The excitation beam from the Fluoromax spectrometer was delivered to the sample through a fused silica optical fibre bundle. Emission was collected and conveyed to the spectrometer via a second optical fibre bundle.

Time-resolved fluorescence

Time-resolved fluorescence spectroscopy was performed using the technique of time-correlated single photon counting, using the same general procedure reported previously.[17] The excitation source was the second or third harmonic of the pulse-picked output of a Ti-Sapphire femtosecond laser system (Coherent, 10 W Verdi and Mira Ti-Sapphire), consisting of pulses of ~200 fs duration at a repetition rate of 4.75 MHz. Fluorescence decays were measured in an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The instrument response of the system was ~50 ps FWHM. Fluorescence decay curves were analyzed using a standard iterative reconvolution method, assuming a multiexponential decay function. Decays were collected at three emission wavelengths and analyzed globally using Edinburgh Instruments' FAST software, i.e. they were fitted simultaneously, with lifetimes, τ_i, as common parameters. The quality of fit was judged on the basis of the reduced chi-square statistic, χ^2, and the randomness of residuals.

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